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From cells to organisms: can we learn about aging from cells in culture?

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Abstract

Can studying cultured cells inform us about the biology of aging? The idea that this may be was stimulated by the first formal description of replicative senescence. Replicative senescence limits the proliferation of normal human cells in culture, causing them to irreversibly arrest growth and adopt striking changes in cell function. We now know that telomere shortening, which occurs in most somatic cells as a consequence of DNA replication, drives replicative senescence in human cells. However, rodent cells also undergo replicative senescence, despite very long telomeres, and DNA damage, the action of certain oncogenes and changes in chromatin induce a phenotype similar to that of replicatively senescent cells. Thus, replicative senescence is an example of the more general process of cellular senescence, indicating that the telomere hypothesis of aging is a misnomer, Cellular senescence appears to be a response to potentially oncogenic insults, including oxidative stress. The growth arrest almost certainly suppresses tumorigenesis, at least in young organisms, whereas the functional changes may contribute to aging, although this has yet to be critically tested. Thus, cellular senescence may be an example of antagonistic pleiotropy. Cross-species comparisons suggest there is a relationship between the senescence of cells in culture and organismal life span, but the relationship is neither quantitative nor direct.

Keywords: Cellular senescence; Immortalization; Fibroblasts; Tumor suppression; Antagonistic pleiotropy; Telomeres; Telomerase; Stress

1. Cells, organisms, and the concept of immortality

Eukaryotic cells, from yeast to mammals, are strikingly similar in the mechanisms by which they execute basic cellular processes such as cell cycle control and DNA repair. Moreover, specialized cells, such as neurons, fibroblasts and secretory epithelial cells, are often remarkably similar in structure and function, even when they originate from very

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different types of multicellular organisms. Needless to say, organisms are exceedingly diverse in the rates at which they age, despite similarities at the level of individual cells. How, then, did the idea evolve that the behavior of cells dissociated from the organism and maintained in culture can provide insights into organismal aging? And, is there reason to believe that this is the case?

1.1. The Carrell experiments

Nearly ninety years ago, a series of experiments by Carrell (1912) spawned the idea that the behavior of individual cells held the key to understanding organismal aging and mortality. Carrell dissociated cells from chicks, placed them in culture, and showed by serial subculture that the cells were apparently capable of proliferating indefinitely. Carrell concluded that cells were inherently 'immortal', and that organismal mortality (and by inference aging) was a consequence of multicellularity. Thus, Carrell equated indefinite cell proliferation with organismal immortality, and lack of cell proliferation with organismal mortality. The logic behind this equivalence was clearly faulty. The cells Carrell cultured were hardly immortal, as they were easily killed by noxious agents or simply poor culture conditions. Moreover, many non-dividing cells (e.g. neurons and mature muscle) are essential for organismal viability, whereas unregulated proliferation is a hallmark of cancer, which kills organisms. Nonetheless, and unfortunately, Carrell's nomenclature persisted, and even today cells that proliferate indefinitely are termed immortal, whereas cells that have a limited proliferative capacity are termed mortal. Parenthetically, Carrell's experiments were also flawed, as it was subsequently discovered that the embryo extract he used to replenish nutrients to his cultures contained fresh viable cells.

1.2. The Hayflick experiments

Nearly fifty years later, Hayflick and Moorehead (1961) challenged the ideas of Carrell by meticulously documenting the fact that normal human fibroblasts have only a limited capacity for proliferation in culture. Since then, many types of normal somatic cells from humans, rodents, birds and several other species have been shown to have a only finite capacity for cell division. The majority of these studies were done using cell cultures. However, a few studies examined proliferative capacity in vivo, and came to a similar conclusion: most normal somatic cells cannot divide indefinitely (reviewed by Stanulis-Praeger, 1987; Campisi et al., 1996). The process that limits cell division has been termed replicative senescence.

Hayflick made two interesting and important connections between the replicative senescence of cells in culture and processes that occur in adult multicellular organisms. First, he noted that most tumor cells appear to proliferate without limitation. This observation generated the idea that replicative senescence might act to suppress tumorigenesis in vivo. Several lines of evidence now strongly support this idea (discussed below, and reviewed by Sager, 1991; Campisi et al., 1996; Yeager et al., 1998; Campisi, 2000a; Reddel, 2000). Hayflick also noted that normal cells appeared aged when they exhausted their replicative potential in culture. He therefore suggested that replicative senescence might also contribute to organismal aging. This idea was, of course, was highly speculative, but

nonetheless solidified the concept that cultured cells can provide insights into the mechanisms of organismal aging and mortality. Support for this idea remains largely correlative (discussed below), and the idea has yet to be tested critically. Nonetheless, it is, I believe, accurate to state that cell cultures have indeed provided important insights into organismal aging, but not, generally, in the direct way implied by these early studies.

2. Replicative senescence, tumor suppression and aging

Replicative senescence is the progressive decline in the ability to proliferate that is an intrinsic property of most normal somatic cell populations. Replicative senescence is limited to cells that have the ability to divide in vivo, and hence does not apply to post-mitotic cells such as mature neurons or muscle. During replicative senescence, cells sense the number of division they have completed, not chronological time. There is a substantial stochastic component to replicative senescence: cells in a given population lose division potential with roughly exponential kinetics, eventually reaching a point at which all cells in the population are senescent and incapable of further cell division. However, the number of doublings at which a cell population reaches complete senescence depends on the cell type and the donor species, age and genotype (reviewed by Stanulis-Praeger, 1987; Campisi et al., 1996). What is the significance of cellular replicative senescence for a multicellular organism, particularly mammals?

2.1. Tumor suppression

Several lines of evidence suggest that replicative senescence, and the more general process of cellular senescence (discussed below), is a powerful albeit imperfect tumor suppressive mechanism. This evidence can be summarized as follows (reviewed in Sager, 1991; Campisi et al., 1996; Yeager et al., 1998; Campisi, 1999, 2000a; Reddel, 2000). First, the majority of naturally occurring and experimentally induced tumors contain cells that are replicatively immortal or have a substantially extended replicative life span. This indicates that there is strong selection during tumorigenesis for mutations that permit cells to overcome the limit to proliferation imposed by replicative senescence. Second, we now know that certain viral and cellular oncogenes transform cells primarily by allowing them to bypass the senescence checkpoint, thereby conferring replicative immortality or extended replicative life span. Third, the p53 and pRb tumor suppressor proteins are critical for the ability of cells to sense the number of doublings they have completed, and enter and remain in the post-mitotic senescent state. The vast majority of tumor cells, if not all tumor cells, harbor mutations that inactivate one or more component of the p53 or pRb pathways, or both. Finally, there are now several mouse models in which germline inactivation of tumor suppressor genes gives rise to an organism in which mitotic cells fail to undergo replicative senescence. These mice inevitably are cancer-prone, and tend to die of cancer at an early age.

2.2. Aging

The idea that the replicative senescence of cells in culture reflects organismal aging

initially rested on three lines of evidence. First, several groups found an inverse correlation between donor age and the number of doublings at which human fibroblasts senesce (reviewed by Stanulis-Praeger, 1987; Campisi et al., 1996). These data have a large amount of scatter, and this correlation is not always seen (Cristofalo et al., 1998). Optimistically, this correlation may suggest that replicative potential is progressively exhausted during organismal aging. Second, limited inter-species comparisons showed an inverse correlation between species life span and the replicative life span of fibroblast cultures. The most complete study used cells from eight mammalian species (Rohme, 1981), but at least one study included cells from the Galapagos tortoise (Goldstein, 1974). These data suggested there might be overlap in genes that control the replicative life span of cells and those that control organismic life span. Third, cells from humans with premature aging syndromes were observed to senesce after fewer doublings than age-matched controls (Martin et al., 1970), again suggesting overlap in genes that control cellular replicative life span and aging phenotypes in organisms. Finally, cells expressing a senescence-associated marker enzyme are more prevalent in physiologically aged tissue, compared to young tissue (Dimri et al., 1995; Mishima et al., 1999; Pendergrass et al., 1999).

In all these studies, emphasis was placed on cellular replicative potential, the idea being that tissue function declines with age because the capacity for renewal or repair is progressively lost. Certainly, decrements in renewal or repair capacity can compromise tissue function, particularly in the immune system or during wound healing. On the other hand, even very old tissues generally have cells with considerable remaining division potential. Moreover, the scatter in the data that compare cellular replicative potential with donor age (Martin et al., 1970; Schneider and Mitsui, 1976), indicate that, at least within a species, the replicative potential of cells does not correlate well with the age of a tissue or donor. This suggests that, if replicative senescence contributes to aging, it is unlikely that cell division potential is the only, or even the major, factor that compromises tissue function and integrity. We have proposed that the changes in cell function that occur when cells senesce, and the fact that senescent cells persist in tissues, may better explain how replicative and cellular senescence can contribute to aging (discussed further below; Campisi, 1996, 1997, 2000a).

2.3. The senescent phenotype and antagonistic pleiotropy

There is an apparent paradox in the proposed consequences of replicative and cellular senescence: how can the same process be both beneficial (by curtailing tumorigenesis) and detrimental (by contributing to aging) to the organism? One possibility is that cellular senescence is an example of antagonistic pleiotropy. That is, cellular senescence may have evolved to maintain health (relative freedom from cancer) early in life, but may have unselected deleterious effects later in life. Why might this be the case?

We now know that replicative senescence induces a complex phenotype in cells, one that entails much more than a cessation of cell division (reviewed by Campisi et al., 1996; Campisi, 2000a). The major features of the senescent phenotype are: (1) an essentially irreversible arrest of cell division; (2) for some cell types, resistance to apoptotic death; (3) selected changes in differentiated cellular functions. Senescence-associated functional

changes include the secretion of a variety of molecules (proteases, cytokines, growth factors) that can act at a distance within tissues and, in principal, drastically alter the tissue microenvironment (Campisi, 1997, 2000a).

The irreversible growth arrest of senescent cells may be the selected phenotype, ensuring that cells with potentially oncogenic damage (discussed below) are incapable of cell division and hence neoplastic growth. By contrast, the resistance to apoptosis and altered functions of senescent cells may be unselected traits. The former may lead to the accumulation of senescent cells in aged tissues. Perhaps more importantly, the altered function of senescent cells, particularly their secretory activity, may contribute to the decline in tissue function and integrity. Among the molecules secreted by senescent stromal cells are metalloproteinases, inflammatory cytokines and growth factors (reviewed in Campisi et al., 1996; Campisi, 1997, 2000a). These molecules are typically expressed only under special circumstances, for example in response to a wound or infection. Under such circumstances, they transiently disrupt normal epithelial–stromal interactions, which are required for normal tissue function. The constitutive secretion of these molecules by senescent cells may therefore chronically disrupt normal tissue functions. These molecules and the resulting disrupted microenvironment may also promote the proliferation of nearby pre-neoplastic cells, thereby actually contributing to tumorigenesis later in life (Campisi, 1997). Indeed, our preliminary data suggest that senescent fibroblasts stimulate the proliferation of pre-neoplastic epithelial cells, both in culture and in vivo (A. Krtolica and J. Campisi, unpublished). Thus, cellular senescence may a striking example of an antagonistically pleiotropic trait — preventing tumorigenesis early in life, but contributing to tissue dysfunction and the growth of preneoplastic cells later in life.

2.4. Replicative senescence and telomere shortening

The number of divisions at which a cell population senesces varies considerably, depending on the cell type and donor species, age and genotype. For example, under standard culture conditions, human fetal fibroblasts generally senesce after 50–80 doublings, whereas mouse fetal fibroblasts do so after 5–10 doublings. How do cells keep track of the number of doublings they have completed? It is now clear that telomere shortening is a major mechanism by which cells from humans (Chiu and Harley, 1997) and certain other species (see, e.g. Shiels et al., 1999; Lanza et al., 2000; Whikehart et al., 2000), sense their replicative history. However, cells from some species, such as mice and probably other rodents (Carman et al., 1998), almost certainly do not undergo replicative senescence as a consequence of telomere shortening (Wright and Shay, 2000; and discussed further below).

Telomeres consist of a repetitive DNA sequence (TTAGGG in vertebrates) bound by specialized proteins. Telomeres cap the ends of linear chromosomes, thereby preventing chromosome fusions and genomic instability. Owing to the biochemistry of DNA replication, each round of DNA replication leaves 50–200 bases at the $3'$ end of the telomere unreplicated. Some cells circumvent this end-replication problem by expressing the enzyme telomerase. Telomerase is a ribonucleoprotein complex that adds telomeric repeats to chromosomes de novo. Germ cells and many tumor cells, which do not undergo replicative senescence, express telomerase. However, most somatic cells do not express

this enzyme. Hence, telomeres shorten with each division of most somatic cells. The average terminal telomeric restriction fragment (TRF) is 15–20 kb in the human germ line. Human cells proliferate until the average TRF reaches 5–7 kb, at which point they cease division with a senescent phenotype (Harley et al., 1990; Levy et al., 1992). Introduction of telomerase activity into at least some normal human cells prevents telomere shortening as well as replicative senescence (Bodnar et al., 1998; Vaziri and Benchimol, 1998). Thus, at least in human cells, telomere shortening is clearly an important regulator of replicative senescence.

3. Cellular senescence, tumor suppression and aging

In the past several years, it has become apparent that cell proliferation and telomere shortening are not the only inducers of the senescent phenotype (the irreversible growth arrest, resistance to apoptosis and altered cellular functions described above). There is now ample evidence that at least three additional stimuli can induce senescence, with little or no cell division (reviewed by Campisi, 2000a).

First, certain types of DNA damage induce normal mammalian cells to undergo a senescence arrest (Di Leonardo et al., 1994; Robles and Adami, 1998; Chen et al., 1998). This includes oxidative damage, which may not be limited to DNA, and double strand DNA breaks.

Second, the expression of certain oncogenes (e.g. activated RAS or RAF), or supraphysiological mitogenic signals (e.g. overexpression of E2F1), induce senescence, rather than apoptosis or transformation, in normal human and mouse fibroblasts (Serrano et al., 1997; Zhu et al., 1998a; Dimri et al., 2000). These findings support the idea that the senescence response is a failsafe mechanism that prevents normal cells that experience potentially oncogenic insults from proliferating.

Third, genetic or pharmacological manipulations that open or decondense chromatin (loss of heterochromatin) induce a senescent phenotype in human and mouse cells (Ogryzko et al., 1996; Jacobs et al., 1999), and accelerate aging in yeast (Kim et al., 1999a; Guarente, 2000). In mammalian cells, locus-specific heterochromatin may limit the expression of tumor suppressor genes. For example, the chromatin remodeling protein BMI-1 suppresses expression of the p16 tumor suppressor (which controls pRb); cells that overexpress BMI-1 have an extended replicative life span, whereas loss of BMI-1 causes premature cellular senescence (Jacobs et al., 1999). Recent studies in yeast suggest that heterochromatin, energy metabolism, recombinational DNA damage, and aging may be linked. The yeast SIR2 protein is required for the heterochromatinization of certain loci. SIR2 also suppresses illegitimate recombination at selected loci, requires NAD for its activity (thereby sensing energy status), and mediates the life span extension caused by caloric restriction (Lin et al., 2000). The link between energy status, chromatin, recombination and cellular aging will undoubtedly be more complex in multicellular organisms, particularly mammals. Nonetheless, there are good reasons to believe that the non-telomere based processes of energy metabolism, chromatin status and recombination will also be important in mammalian aging, and will operate at the level of individual cells (Campisi, 2000b).

Taken together, these findings indicate that replicative senescence (caused by telomere shortening) is a specific example of a much broader process termed cellular senescence. Moreover, these findings suggest that it is cellular senescence, rather than replicative senescence per se, that may be important in organismal aging.

4. The telomere/telomerase hypothesis of aging

The findings that telomere shortening triggers replicative senescence in human cells, and that telomeres shorten in several cell types during human aging, has led to the hypothesis that telomere shortening is an important determinant of human aging (Harley, 1997; Hodes, 1999). How valid is this hypothesis, and is it applicable to other species?

4.1. Telomeres

There is little doubt that telomere attrition causes the replicative senescence of human, bovine and other cells. On the other hand, as discussed above, cells can acquire senescent characteristics by telomere-independent mechanisms. Even in replicating human cells, there is evidence that telomere length does not necessarily determine whether cells senesce (Ouelette et al., 2000). It has been suggested that human cells are unique among primates in having relatively short (~10–15 kb) telomeres in somatic tissues (Kakuo et al., 1999), raising the possibility that telomere-based senescence may be peculiar to cells from long-lived species. Certainly, telomere-driven senescence is not universal among species. Mouse cells undergo proliferative arrest after 5–10 doublings in culture, despite very long telomeres and, in many cases, expression of telomerase (reviewed in Sherr and DePinho, 2000; Wright and Shay, 2000). Moreover, among species, there is no correlation between telomere length (whether in the germ line or somatic tissues) and mean or maximum organismal life span. This lack of correlation is perhaps best illustrated by *Mus musculus* and *Mus spretus*. These closely related, inter-fertile organisms have quite similar life spans, but markedly different telomere lengths (Coviello-McLaughlin and Prowse, 1997; Zhu et al., 1998b). Wright and Shay (2000) recently proposed that short-lived species may have less need than long-lived species for telomere based senescence. Whatever the case, it appears evident that the relationship between telomere length and aging is indirect at best, and non-existent in some cases. Telomere length can certainly be a useful biomarker of aging in some proliferative cell types or tissues (e.g. T cells or aortic endothelium; Effros, 1998; Chang and Harley, 1995) in those species (such as humans) in which cells undergo telomere-based senescence. However, there is at present no evidence that telomere length per se contributes to organismal aging. Rather, cellular senescence (driven in part by telomere shortening) may contribute to organismal aging, although, as noted earlier, this idea awaits critical testing.

4.2. Telomerase

A common popular corollary of the telomere hypothesis of aging suggests that telomerase expression may postpone aging and promote longevity. There are several reasons to doubt that this is the case. First, cells that do not undergo replicative senescence owing

to expression of telomerase nonetheless undergo cellular senescence in response to DNA damage or oncogene expression (Jiang et al., 1999; Morales et al., 1999; Wei et al., 1999). Moreover, some cells (e.g. Syrian hamster fibroblasts, human T lymphocytes) express telomerase, but nonetheless undergo replicative senescence. In some cases (human T cells), senescence occurs with attendant telomere shortening, despite telomerase expression (Carman et al., 1998; Effros, 1998; Russo et al., 1998; Hodes, 1999). These and the other findings (Kim et al., 1999b; Evans and Lundblad, 2000; Smogorzewska et al., 2000) indicate that telomere-associated proteins regulate telomerase access to the telomere, and that telomerase alone can be insufficient to maintain telomere length and prevent replicative senescence. Second, although most somatic tissues in adult humans are completely devoid of telomerase activity, the adult tissues of some species (chickens, mice, and certain marine vertebrates and invertebrates, for example) express substantial levels of telomerase (Prowse and Greider, 1995; Klapper et al., 1998a,b; Venkatesan and Price, 1998). Among these species, there is no obvious correlation between telomerase expression in somatic tissues and species-specific life span. Third, although telomerase is essential for the maintenance and health of highly proliferative tissues in mice (Blasco et al., 1997; Lee et al., 1998) and humans (Mitchell et al., 1999), telomerase expression also favors the development cancer in cells harboring certain oncogenic mutations (Blasco et al., 1997; Hahn et al., 1999a,b).

4.3. Telomeres, telomerase and aging

Taken together, the current data on telomere length and telomerase expression strongly suggest there is no basis for a general telomere/telomerase hypothesis of aging. Certainly, telomere length and telomerase may play a non-exclusive role in the aging of organisms that exhibit telomere-dependent replicative senescence. However, whether this is case depends on whether the hypothesis that cellular senescence contributes to aging is correct. Moreover, while short dysfunctional telomeres clearly contribute to the atrophy or development of cancer in proliferative tissues (Blasco et al., 1997; Lee et al., 1998; Mitchell et al., 1999), there is as yet no evidence that maintenance of telomere length or elongation of functional telomeres improves the health or longevity of complex multicellular organism such as mammals. Thus, the ‘telomere hypothesis of aging’ is a misnomer. It is essentially an oversimplified statement of the cellular senescence hypothesis of aging, which of course has yet to be critically validated.

5. Telomere-independent senescence, stress, and species-specific life span

Since neither telomere length nor the presence of telomerase predicts the replicative life span of cultured cells, much less the life span of organisms, why then do cultured cells from species with relatively short life spans cease division more readily than cells from species with longer life spans?

One possibility is that cells vary in their sensitivity to culture conditions, which may cause a telomere-independent damage arrest (Sherr and DePinho, 2000; Wright and Shay, 2000). The most obvious culprit is oxygen — ambient oxygen concentrations (~20%) far exceed the oxygen concentrations experienced by cells in vivo (which typically vary from

2 to 5%). Thus, standard culture conditions expose cells to oxidative stress. It is well established that culturing human fibroblasts in 2–3% oxygen allows them to achieve 20–30% more population doublings, compared to the population doublings achieved under standard 20% oxygen (Balin et al., 1984; Saito et al., 1995; Chen et al., 1995). By contrast, our preliminary results suggest that mouse fibroblast cultures achieve 2- to 3-fold greater population doublings when cultured in 3% oxygen, compared to 20% oxygen (Parrinello, S. and Campisi, J., unpublished). These findings suggest that mouse cells are much more sensitive than human cells to the oxidative stress commonly encountered during routine cell culture. Moreover, they raise the possibility that species-specific differences in cellular replicative life span (under standard culture conditions) may reflect differences in the ability of cells to tolerate oxidative stress, rather than telomere-limited replicative potential. Thus, at least among the mammalian species studied by Rohme (1981), the differences in replicative life span shown by the cell cultures may more accurately reflect species-specific differences in the ability of cells to proliferate under relatively high (20%) oxygen concentrations.

In support of this idea, cellular resistance to chemically induced oxidative stress has been shown to correlate with organismal life span among eight mammalian species (Kapahi et al., 1999). Unfortunately, there was little overlap in the species studied by Rohme (1981) and those studied by Kapahi et al. (1999). Moreover, one of the few genetic manipulations that extended the life span of a mammal (mice) conferred resistance to oxidative stress on fibroblasts cultured from the animals. Germline inactivation of the p66/shc gene, which encodes an adaptor protein that functions in transmembrane signal transduction, extended the life span of mice by about 30% (Migliaccio et al., 1999). This magnitude of life span extension is similar to that achieved by caloric restriction. Embryo fibroblasts cultured from p66/shc -/- mice showed a corresponding increase in resistance to chemically induced oxidative stress.

Thus, replicative life span of cells, at least under standard tissue culture conditions, may better reflect species-specific differences in stress tolerance, which in turn may predict organismal life span. It has been noted that the relationship between the replicative life span of cultured fibroblasts and average species life span might reflect differences in body size, which also correlates with organismal life span. However, there are many exceptions to general rule that small organisms are shorter lived than large organisms (Miller and Austad, 1999). Moreover, avian species are notorious exceptions to the body size-organismal life span correlation, having somewhat longer life spans than expected from body size. Interestingly, fibroblast cultures from chickens and other avian species have a relatively long replicative life span (30 or so doublings) (see Venkatesan and Price, 1998) in comparison to cultures from similarly sized mammals (such as rabbits, cells from which generally senesce after 15 or so doublings) (Rohme, 1981). Unfortunately, there is very little data on the replicative life spans or senescence characteristics of cells cultured from species other than mammals or birds, even though cells from organisms such as fish have been cultured for quite some time (Wolf and Mann, 1980; Zhu and Sun, 2000). That being the case, the data available thus far suggest that the more general process of cellular senescence, which occurs in response to many stimuli, including oxidative stress, may provide a general reflection of organismal life span.

6. Summary

In summary, cell cultures can provide information and molecular handles on the intrinsic sensitivity of cells to oxidation and other stresses, the ability to sense and handle DNA damage and telomere dysfunction, and the response to oncogenic stimuli. This information, in turn, is very likely related to species life span.

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